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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

In re the application of

HAUER et al.

Serial No. 10/031,291

Filed: January 17, 2002

) MAIL STOP APPEAL BRIEF

) Group Art Unit: 1652

) Examiner: Pak

For: ELECTRON DONOR SYSTEM FOR ENZYMES AND ITS USE IN THE  
BIOCHEMICAL CONVERSION OF SUBSTRATES

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BRIEF ON APPEAL

Sir:

This appeal is from the examiner's final office action mailed on June 3, 2004.

Applicants' notice of appeal was received on October 25, 2004.

REAL PARTY IN INTEREST

The real party in interest is BASF Aktiengesellschaft, of Ludwigshafen, Germany.

Reel/Frame 012696/0020, recorded on January 17, 2002.

RELATED APPEALS AND INTERFERENCES

To appellants' knowledge and belief, there are no interferences or other appeals  
which will directly affect or be directly affected by or have a bearing on the Board's

HAUER et al., Serial No. 10/031,241

decision in this application.

#### STATUS OF THE CLAIMS

Claims 1-22 currently are pending. Claims 1-10, 13-15 and 19-22 have been withdrawn from consideration by the examiner. Claims 11-12 and 16-18 are rejected.

#### STATUS OF THE AMENDMENTS

The claims have not been amended subsequent to the final office action mailed on June 3, 2004.

#### SUMMARY OF THE INVENTION

The present invention relates to a novel electron donor system for enzymes with redox properties, and to the use thereof in enzyme-catalyzed oxidation reactions such as, in particular, the preparation of  $\omega$ -hydroxylated fatty acids. The invention additionally relates to an improved detection method for fatty acid monooxygenases, to bioreactors and to test kits in which the electron donor system can advantageously be employed. (Specification, page 1, lines 5-11).

#### ISSUES

Whether claims 11-12 and 16-18 are obvious over Eastbrook et al. in view of Sargeson et al.

#### GROUPING OF CLAIMS

The claims have not been argued separately.

#### ARGUMENT

The following legal authorities are relied on in the following arguments in the

HAUER et al., Serial No. 10/031,241

order in which they are cited:

MPEP § 2143;

*In re Nomiya, Kohisa, and Matsumura*, 509 F.2d 566, 184 USPQ2d 607 (CCPA 1975);

### REJECTION

Claims 11-12 and 16-18 are rejected under 35 USC § 103(a) as being unpatentable over Estabrook et al. in view of Sargeson et al. The examiner stated that the difference between Estabrook et al. and the instant invention is that Estabrook et al. does not teach the method of hydroxylating fatty acids using Zind dust as the source of electrons. However, the examiner believes it is well-known in the art that Zinc serves as reductants and Sargeson et al. teach that the caged metal complex have uses as inert oxidation-reduction reagents in inorganic and organic oxidation and reduction reaction (column 4). Therefore, the examiner believes it would have been obvious to one having ordinary skill in the art at the time the claimed invention was made to use the Zn/Co sepiolite complex of Sargeson et al. to hydroxylate fatty acids. The motivation to combine or modify the references the examiner sets forth seems to be: "An alternative to using electrodes as a source of electrons is attractive since irreversible adsorption of protein constituents leading to electrode fouling and protein denaturation may occur." (Office action, page 4, last paragraph).

To establish a *prima facie* case of obviousness, three basic criteria must be met.

HAUER et al., Serial No. 10/031,241

First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. MPEP § 2143.

Applicants believe the examiner has not met the above the requirements for establishing a *prima facie* case of obviousness. Specifically, applicants believe the examiner engages in hind-sight reasoning. There must be a reason apparent at the time the invention was made to a person of ordinary skill in the art for applying the teaching at hand, or the use of teaching as evidence of obviousness will entail prohibited hindsight. *In re Nomiya, Kohisa, and Matsumura*, 501 F.2d 566, 184 USPQ 607 (CCPA 1975).

The teachings of Estabrook et al. differs from the present invention by the electron source used for performing the enzymatic reaction. Estabrook et al. applies a platinum electrode as electron source. Eastabrook et al. has observed for the hydroxylation of lauric acid with the P450 enzyme BM3 conversion rates of 110 nm/min/nm P450 when applying the Pt-electrode as electron source. This corresponds to a relative reaction rate of about 12% if compared to the reaction applying NADPH instead of the Pt-electrode (see Table I on page 46 of Estabrook et al.). Estabrook et al. makes no suggestions for alternative approaches to improve further the reaction rate for the NADPH independent reaction.

HAUER et al., Serial No. 10/031,241

Would a skilled person aware of the teaching of Estabrook et al. Sargeson et al. and Creason et al. have been motivated to modify the teaching of Estabrook to teach each and every element of the present invention? Applicants believe the answer is no.

While it is correct that Creaser et al. discloses a reaction between cobalt sepulchrate and Zn dust this reference does not teach or suggest that the system might also be applied, under physiological conditions, in enzyme-catalyzed hydroxylation reactions of fatty acids of the claimed type. The suggested treatment of said complex with zinc dust and HCl (see Sargeson, column 4, lines 20-23) does not represent physiological conditions applicable to enzyme-catalyzed reactions. Therefore, applicants believe the examiner used hindsight reasoning.

Applicants refer the examiner to further experimental work done by the present inventor. Similar to the experiments done by Estabrook and summarized in Table I of the Estabrook, the present inventor has compared reaction rates obtained for the BM-3 mutant F87A (also mentioned in the experimental part of the present specification) under different conditions. To measure enzyme activity via an optical test the artificial substrate 12-pNCA (disclosed on page 31 of the specification) was used as enzyme substrate. Enzyme activity was measured in a first experiment in the presence of NADPH as electron source and in a second experiment (according to the invention) in the presence of Zn dust and Co (III) sepulchrate as mediator. Setting the reaction rate of the first experiment as 100% the present inventor surprisingly observed for the reaction according to the second experiment (reaction according to the present

HAUER et al., Serial No. 10/031,241

invention) a relative reaction reate of about 22%.

From said data it becomes clear that according to the present invention (if compared with Estabrook et al. ) a relative rate approximately double as high as that observed by Estabrook et al. for their system (Pt-electrode/Co (III) sepulchrates) is observed. This shows that the electron donor system of the present invention is more suitable for enzyme reactions of the claimed type as it allows improved reaction rates. A skilled person aware of the teaching of the prior art would never have expected such a significant improvement.

Attached is a table summarizing the above results.

For the reasons expressed above, it is urged that the prior art references cited by the examiner either singly or in combination fail to anticipate or suggest the present invention as defined by the amended claims. Accordingly, a *prima facie* case of obviousness has not been established by the examiner, and the rejection under 35 USC § 103 should be withdrawn.

### CONCLUSION

For the foregoing reasons, it is respectfully submitted that reversal of the examiner's rejection of all claims is in order.

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HAUER et al., Serial No. 10/031,241

Respectfully submitted,

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HAUER et al., Serial No. 10/031,241

### **APPENDIX**

1. An electron donor system for transferring electrons to enzymes with redox properties, wherein the system comprises an inorganic, non-electrode-bound source of electrons and a mediator which is able to transfer electrons from the source of electrons to the enzyme.
2. An electron donor system as claimed in claim 1, wherein the enzyme is a cytochrome P450-containing enzyme.
3. An electron donor system as claimed in claim 2, wherein the enzyme is a mono oxygenase (E.C. 1.14).
4. An electron donor system as claimed in claim 1, wherein the mediator has a standard normal potential in the region of less than about -0.4 V.
5. An electron donor system as claimed in claim 1, wherein the mediator is selected from cobalt(III) sepulchrates, methylviologen, neutral red, riboflavin, ruthenium triacetate, FMN and FAD.
6. An electron donor system as claimed in claim 1, wherein the source of electrons is a metal with a lower standard normal potential than the mediator.
7. An electron donor system as claimed in claim 6, wherein the source of electrons is metallic zinc.
8. An electron donor system as claimed in claim 1, selected from the systems:
  - Zn/cobalt(III) sepulchrates and
  - Zn/neutral red.



HAUER et al., Serial No. 10/031,241

9. A method for the enzymatic transfer of oxygen to a hydrocarbon-containing hydrogen donor molecule, which comprises incubating the hydrogen donor molecule in a reaction medium comprising the oxygen-transferring enzyme and an electron donor system as claimed in claim 1 in the presence of oxygen under reaction conditions.

10. A method as claimed in claim 9, wherein the hydrogen donor molecule is selected from compounds of the formula



in which

R is an alkyl radical with 8 or more carbon atoms, and

X is a polar group capable of forming hydrogen bonds, preferably a carboxyl, amide, nitrile, sulfate, sulfone, amine or hydroxyl group.

11. A method for the enzymatic production of terminally or subterminally (position  $\omega$ -1 to  $\omega$ -4) hydroxylated fatty acids, which comprises

a) converting a hydroxylatable fatty acid or fatty acid derivative in the presence of an electron donor system as claimed in claim 1 using a cytochrome P450 mono oxygenase and oxygen; and

b) isolating the hydroxylated product(s).

12. A method as claimed in claim 11, wherein the  $\omega$ -hydroxylatable fatty acid derivative is selected from terminally saturated, branched or unbranched fatty acids with more than 10 carbon atoms, in particular  $C_{12}$ - $C_{30}$  fatty acids.

HAUER et al., Serial No. 10/031,241

13. A method as claimed in claim 9, wherein the enzyme is a cytochrome P450 mono oxygenase selected from:

- a) the wild-type enzyme which can be isolated from *Bacillus megaterium* (DSM 32T); or
- b) a mutant, which can be obtained by amino acid substitution in at least one of positions 26, 47, 72, 74, 87, 188 and 354, of the wild-type enzyme (SEQ ID NO:35).

14. A method as claimed in claim 13, wherein a single mutant selected from F87A, F87V, L188K, V26T, R47F and V26T is employed.

15. A method as claimed in claim 13, wherein the mutant has in position 87 the mutation F87A or F87V and at least one other of the following mutations: L188K, A74G, R47F and V26T.

16. A method as claimed in claim 11, wherein the electron donor system is zing/Co(III) sepulchrate.

17. A method as claimed in claim 11, wherein at least stage a) is carried out in the presence of chloride ions.

18. A method as claimed in claim 11, wherein at least stage a) is carried out in the presence of a hydrogen peroxide-cleaving enzyme.

19. A bioreactor for use producing  $\omega$ -hydroxylated fatty acids, which comprises immobilized monooxygenase and an electron donor system as claimed in claim 1 in a liquid reaction medium.

HAUER et al., Serial No. 10/031,241

20. A detection method for fatty acid monooxygenases, which comprises
- a) Incubating an analyte suspected of having enzymatic activity with an  $\omega$ -hydroxylatable fatty acid or fatty acid derivative which has a terminal chromophore or fluorophore which can be eliminated, in the presence of an electron donor system as claimed in claim 1; and
  - b) determining the elimination of the chromophore or fluorophore qualitatively or quantitatively.
21. A method as claimed in claim 20, wherein the conversion is carried out in the presence of a hydrogen peroxide-cleaving enzyme and, where appropriate, in the presence of chloride ions.
22. A test kit comprising an electron donor system as claimed in claim 1.

JUL 20 2005 4:20PM

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	P450 enzymes	electron source	mediator	substrate	reaction rate [eq/min]*	relative rate
Estabrook et al.	BM-3	NADPH	-	lauric acid	900	100 %
Estabrook et al.	BM-3	electrolysis/Pt-electrode	Co(III)sep	lauric acid	110	12 %
Inventor	BM-3 F87A	NADPH	-	12-pNCA	574 ± 28	100 %
Inventor	BM-3 F87A	zinc dust	Co(III)sep	12-pNCA	125 ± 6	22 %

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JUL 20 2005 4:20PM

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P. 16

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HAUER et al.

Serial No. 10/031,241

NOTICE OF APPEAL



January 25, 2005

HAUER et al.

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